_**ORIGINAL RESEARCH**_

# Quantitation of meiosis activating sterols in human follicular fluid using HPLC and photodiode array detection

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ABSTRACT: A chromatographic assay for 4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol (FF-MAS), and its reduced species, 4,4-dimethyl-5α-cholesta-8,24-triene-3β-ol (T-MAS), has been established for analysis of human follicular fluid (huFF). The assay also quantifies lanosterol, free cholesterol and progesterone. It was established using a pool of more than 100 individual follicular fluids from women undergoing *in vitro* fertilization treatment. Both FF-MAS and T-MAS were found in huFF, and can be quantified with HPLC equipped with photodiode array (PDA) detection. The examination wavelength for each analyte was chosen at the absorption maximum between 200 and 300 nm. Spike-recovery experiments revealed mean recoveries of 91 ± 7.3% for lanosterol, 103 ± 5.1% for FF-MAS, 104 ± 5.5% for T-MAS, 103 ± 4.5% for free cholesterol and 85 ± 5.1% for progesterone. The lower recovery value for progesterone was due to a sub-optimal extraction procedure for this particular analyte, as indicated by re-extraction. The minimum amounts of FF-MAS required for quantification were 4 ng/mL and 23 ng/mL for T-MAS and lanosterol. FF-MAS was assayed to approximately 1.6 μM. T-MAS and lanosterol was assayed to about half of this value. No esterification of either MAS or lanosterol could be detected in huFF. Less than 10% of cholesterol was underivatized cholesterol, as more than 10 times the amount of free cholesterol could be assayed after extended saponification. This method can be used for evaluating the accumulation of MAS in huFF and its correlation to oocyte quality and fertilization parameters in *in vitro* fertilization programmes. Copyright © 1999 John Wiley & Sons, Ltd.

## INTRODUCTION

Sterol transportation and metabolism in mammalian gonads is crucial for steroidogenesis, where free cholesterol serves as the substrate for steroidogenic enzymes. Most cholesterol utilized for this purpose is generally believed to be derived from lipoproteins (Christie *et al.*, 1979; Gwynne and Strauss, 1982, Grummer and Carroll, 1988). However, cholesterol synthesis *de novo* has been reported in ovine (Douglas *et al.*, 1978), porcine (Barañao and Hammond, 1986) and bovine (Spicer *et al.*, 1996) granulosa cells, rat ovaries (Bai *et al.*, 1973) or luteal cells (Christie *et al.*, 1979; Azhar *et al.*, 1985), and isolated rabbit follicles (Mills and Savard, 1972), and it appears that steroidogenesis can proceed to some extent by the *de novo* pathway alone (Schuler *et al.*, 1981).

The discovery that two gonad-derived 4,4-dimethyl-sterols have the ability to induce meiotic resumption *in*

*vitro* (Byskov *et al.*, 1995) has led to novel insight into the importance of ovarian sterol synthesis *de novo* with respect to follicular and oocyte maturation. These so-called meiosis activating sterols (MAS) are intermediates in the cholesterol biosynthetic pathway (Aoyama *et al.*, 1984). It was shown that substantial amounts of 4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol (FF-MAS) accumulate in human follicular fluid (huFF), and the reduced species, 4,4-dimethyl-5α-cholesta-8,24-triene-3β-ol (T-MAS), accumulates in bull testis (Byskov *et al.*, 1995). Intermediate steps in the conversion of squalene into cholesterol involve the C-14 demethylation of lanosterol by P45014DM, which in turn produces (FF-MAS) (Aoyama *et al.*, 1984). P45014DM is encoded by the evolutionary highly conserved CYP51 gene (Strömstedt *et al.*, 1996). Any cell that produces cholesterol *de novo* is therefore able to produce MAS. Recently, high levels of a variant form of CYP51 mRNA has been detected in testes and ovaries of rats (Strömstedt *et al.*, 1998). Thus, a gonad-specific regulatory pathway for intermediates in the cholesterol biosynthesis pathway may exist.

The accumulation of 4,4-dimethylsterols has been reported in human serum (Miettinen, 1968; Lund *et al.*, 1990), rat serum (Miettinen, 1969) and organs (D'Hollander and Chevallier, 1968), but no specific amounts were given of those sterols which proved to be involved

in meiosis activity. A protocol for FF-MAS and T-MAS isolation has been described (Byskov *et al.*, 1995), but no assay protocol for the quantification of MAS has been developed. Here we present a simple two-step chromatographical analysis for MAS, its precursor lanosterol and the products cholesterol and progesterone in huFF. The analysis was employed in order to determine the concentrations of MAS in huFF as well as the extent of esterification of MAS.

## EXPERIMENTAL

**Materials and reagents.** HuFF was obtained from the female of couples undergoing treatment for infertility by *in vitro* fertilization and embryo transfer after gonadotrophin stimulation. Patients were given an ovulatory dose of 10,000 IU hCG. After 36 h the follicular fluid (FF) was aspirated and frozen immediately after oocyte recovery; FF's contaminated with blood were excluded. The FF pool used for preparative purification of FF-MAS represents material from several follicles from a large number of patients and amounted to approximately 3 L (>500 follicles). A pool of approximately 600 mL (>100 follicles) was used for analytical runs.

A bull testis was obtained from a slaughterhouse, transported on dry ice and frozen at −20°C prior to use as a source of T-MAS (Byskov *et al.*, 1995).

Isopropanol (Fisher, analytical grade), n-heptane (BDH, HiPerSolv®) and acetonitrile (Baker, HPLC-reagent) were used for mobile phases in chromatographic runs.

**Preparative purification of MAS.** HuFF (roughly 3 L) was extracted in a double volume of 75% n-heptane:25% isopropanol (v/v) for 24 h by stirring in large glass containers in the dark. The organic solvent was separated from the water phase by decantation and subsequently dried under reduced pressure at 40°C using a rotation evaporator. The extract was washed twice in re-distilled water and reconstituted in approximately 2 mL n-heptane. Aliquots of 150 µL were loaded onto a ChromSpher® Si, 5 µm, 250 × 10 mm column, running in mobile buffer [v/v: 99% n-heptane (BDH):1.0% isopropanol (Fisher)] at 5.00 mL/min at 28°C. Fractions between 5.5 and 7.5 min were collected, pooled and dried again. The pool was reconstituted in approximately 2 mL acetonitrile and aliqouts of 144 µL were loaded onto a LiChrospher® RP-8 EC, 5 µm, 250 × 4.6 mm HPLC column, running in mobile phase [v/v: 92.5% acetonitrile (BDH):7.5% water] at 1.00 mL/min, 40°C. FF-MAS was collected between 8 and 9.75 min.

A 90 g sample of tissue from a bull testicle was cut into small pieces no larger than 5 mm and freeze-dried. The material was subsequently crushed to fine powder in a mortar and extracted in about 500 mL 75% n-heptane:25% isopropanol (v/v) for several hours by vigorous shaking in a darkened glass container. The organic solvent was separated from the tissue sample by centrifugation and decantation and subsequently dried and treated as described above for FF. Only the reversed-phase separation differed, as T-MAS was collected between 10 and 11.25 min.

These purification steps produced 1.4 mg FF-MAS and 3.5 mg T-MAS. Their purity was checked by comparing their absorption

patterns between 200 and 300 nm with a reference characterized by Byskov *et al.* 1995. Prepared samples were weighed with an accuracy of ±10% on a Sartorius RC 210D.

**Assay for 4,4-dimethylsterols.** Frozen samples of huFF were diluted to a final volume of 1.00 mL in PBS placed in 4 mL teflon-capped glass vials. 100 µL of 0.30 M $NaH_2PO_4$, pH = 1.0 and 2.50 mL 75% n-heptane (BDH):25% isopropanol (Fisher) were added, and the solution was subjected to vigorous mixing (1800 rpm, amplitude 4 mm) for 90 min. The organic phases were separated from the water phase by centrifugation (2000 *g*, 15 min), aspirated and placed in tubes with a tapered end. Secondary extractions were performed on the remaining water phase in order to analyse the extent of removal of analytes by the first round of extraction.

Saponifications were carried out according to a method previously described by Tsui 1989, but with a slight modification. Briefly, 200 µL huFF were added to 800 µL 0.50 M KOH in EtOH and heated to 37°C for between 5 min and 45 h. After alkaline hydrolysis, the samples were adjusted to pH = 3.5–4.0 with 5 M HCl and treated as above.

Recovery studies were made by adding known amounts of an analyte mixture (lanosterol, FF-MAS, T-MAS, cholesterol and progesterone) in n-heptane to the 1000 µL samples of huFF before treatment as indicated above. The amount of each analyte was approximately 100% of the amount assayed in the pure sample. Recoveries were calculated by comparing the samples to a dilution series of pure analyte mixture.

The organic phases of all samples were dried under reduced pressure at 40°C in a vacuum centrifuge (1 mbar, 25 min), reconstituted in 115–125 mg n-heptane (169–183 µL) on a precision balance and separated by straight-phase HPLC (Waters 510/717/996, Hedehusene, Denmark). First, 144 µL were loaded onto a ChromSpher® Si, 5 µm, 250 × 4.6 mm HPLC column, running in mobile buffer [v/v: 99.5% n-heptane (BDH):0.5% isopropanol (Fisher)] at 1.00 mL/min, 28°C. The 4,4-dimethyl-sterols of interest eluted between 9 and 10.5 min, cholesterol at 19 min and progesterone at 23.4 min [Fig. 1(a)]. The components to be analysed in the first separation were detected by ultraviolet absorption at the indicated wavelengths and identified by their characteristic absorption spectra between 200 and 300 nm [FF-MAS, 248.8 m; cholesterol, 200.0 nm; and progesterone, 234.7 nm; Fig. 1(a–c)]. Eluted material between 8 and 11 min was collected and subjected to reversed-phase separation. The fractions were dried as above and separated by reversed-phase HPLC: the samples were reconstituted in 130–140 mg acetonitrile (Baker) (160–175 µL), of which 144 µL were loaded onto a LiChrospher® RP-8, 5 µm, 250 × 4.6 mm HPLC column, running in mobile buffer [v/v: 92.5% acetonitrile (BDH):7.5% water] at 1.00 mL/min, 40°C. FF-MAS (8.5 min), T-MAS (10.5 min) and lanosterol (11.4 min) eluted as single peaks, as determined by ultraviolet absorption at 200 nm. The purities were checked by spectral analysis at various points of the eluting peaks (Fig. 2).

*Quantitation*: lanosterol, free cholesterol and progesterone were quantified by comparing them to standards obtained by weighing material from commercial samples (lanosterol, L-5768, Sigma, MO, USA, cholesterol, C-6760, Steraloids, NA, USA; progesterone, P-0130, Sigma, MO, USA). MAS were quantified by comparing them to samples obtained from the preparative procedures outlined above. The individual chromatograms were
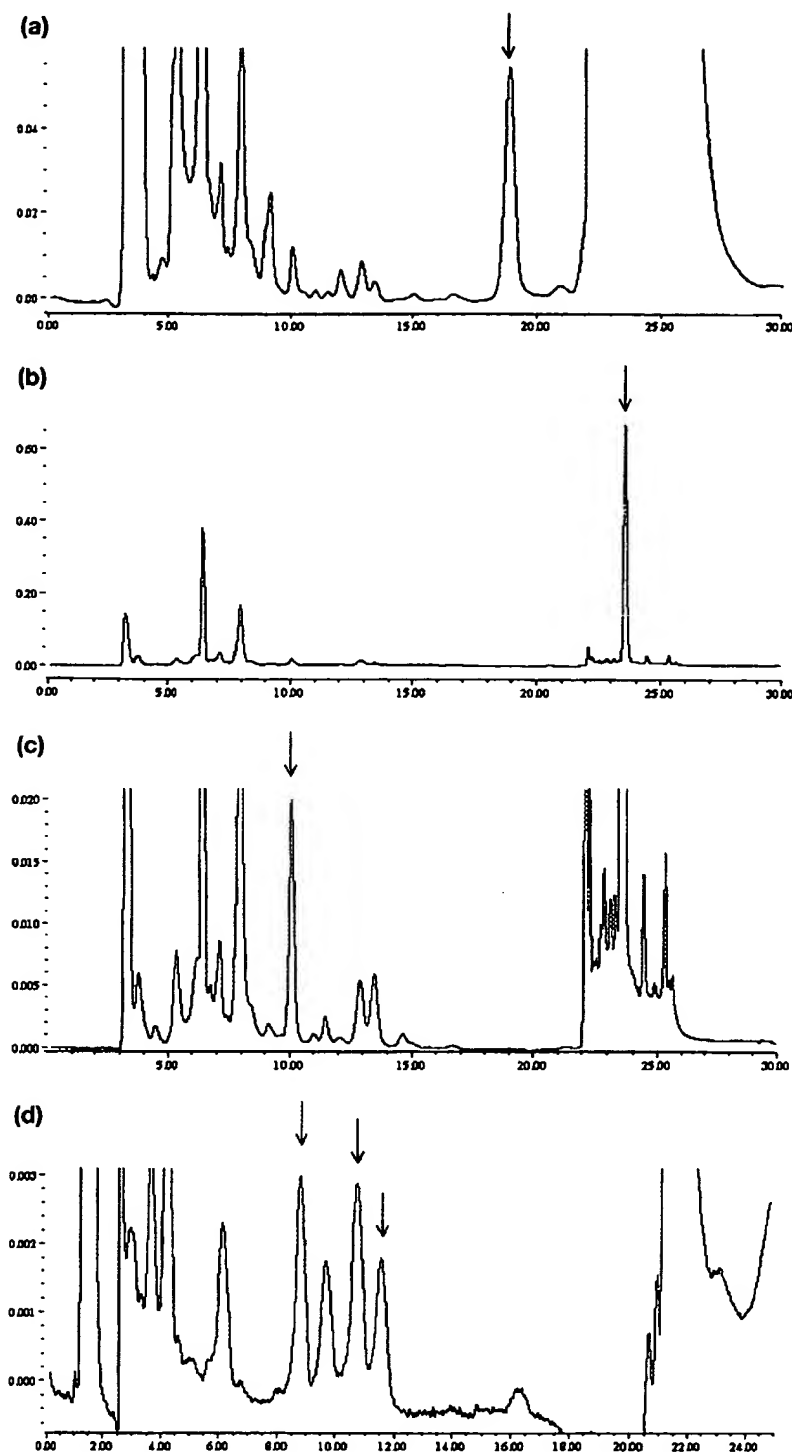
**Figure 1.** Straight-phase chromatograms extracted at 200 nm (a), 234.7 nm (b) and 248.8 nm (c) for huFF and reversed-phase chromatogram extracted at 200 nm (d). Arrows in (a)–(c) indicate retention times for free cholesterol, progesterone and FF-MAS, respectively. Arrows in (d) indicate reversed-phase retention times for FF-MAS (upper left), T-MAS (middle) and lanosterol (lower right).
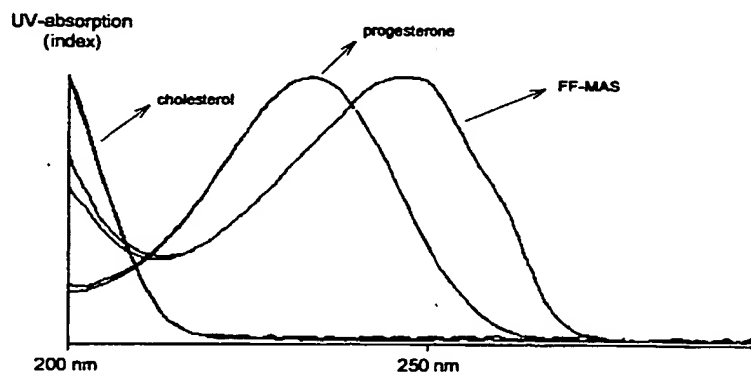
**Figure 2.** Normalized UV-spectra between 200 and 300 nm for analytes assayed in the (first) straight-phase separation. Apical spectra for all analytes in the straight-phase separation are shown as well and appear confluent with spectra extracted from standard injections of cholesterol, progesterone and FF-MAS. Confluency was even more rigorous for reversed-phase analytes (not shown).

evaluated at indicated wavelengths using Millennium<sup>©</sup> software (Waters). The criteria for threshold, band width and baseline identification were chosen by baseline analysis of empty injections in order to avoid any acceptance of artificial bands. The criteria were chosen before analysis and were equal for all runs.

## RESULTS AND DISCUSSION

The PDA option allows for the evaluation of specific analytes at their optimal wavelength, ie where the signal-to-noise ratio is maximal. Figure 2 shows the UV-spectra for standards of all analytes analysed by straight-phase mode, normalized to the maximum absorbance in the 200–300 nm region. FF-MAS, free cholesterol and progesterone-were-sufficiently resolved by straight-phase HPLC separation, using 248.8, 200 and 234.7 nm, respectively, as the evaluation wavelength [Figs 1(a–c) and 2]. Lanosterol and T-MAS were resolved by re-chromatography on reversed phase [Fig. 1(d)]. Comparing spectra from injections of standards with spectra evaluated at the apex of chromatograms obtained by separating huFF revealed an easy identification of FF-MAS, cholesterol and progesterone by straight-phase analysis (Fig. 2).

A linear relationship between the analysed volume of huFF and the assayed amount of analytes is shown for FF-MAS, cholesterol and progesterone [Fig. 3(a)], as well as for lanosterol and T-MAS [Fig. 3(b)]. Samples from the dilution series were treated and analysed in different sample sets. Precision therefore represents total (intra- and interassay) variation. With a maximum standard deviation of 10%, 50 µL is required for the quantification of progesterone, 10 µL for the quantifica-

tion of FF-MAS and 5 µL for the quantification of cholesterol. T-MAS and lanosterol require 50 and 100 µL, respectively, with a 15% standard deviation level. The limits of detection and quantification are given in Table 1.

In order to validate the assay, spike-recovery tests and re-analysis of the water phase were performed. In general, a level close to 100% recovery was found for sterol analytes for which the extraction procedure was optimized, whereas progesterone could be recovered to about 85% (Fig. 4). The latter was probably due to a non-optimal apolar extraction procedure for progesterone, which could be explained by the fact that progesterone displays a lower lipophilicity as compared to the sterols. Re-extraction experiments corresponded fairly well with this, in that no 4,4-dimethylsterols could be detected in a second round of analysis whereas approximately 6% progesterone was measured (Table 2).

Saponification showed that less than 10% of total cholesterol in huFF is represented as underivatized (free) cholesterol, whereas 4,4-dimethylsterols appear non-esterified (Fig. 5).

The total amount of MAS in pre-ovulatory huFF exceeds 2 µM. Contrary to earlier reports (Byskov *et al.*, 1995), FF-MAS and T-MAS persist in huFF. The assay presented here is suitable for analysis of MAS, progesterone and MAS/cholesterol indices in as little as 100 µL huFF. The method may be used for evaluation of MAS accumulation in huFF and its correlation to oocyte quality and fertilization parameters in *in vitro* fertilization programmes. Data from our laboratory show that the assay is suitable for MAS analysis in FF from other mammalian species as well (data not shown). Moreover, the analysis provides an appropriate method for the

**a.**



**b.**
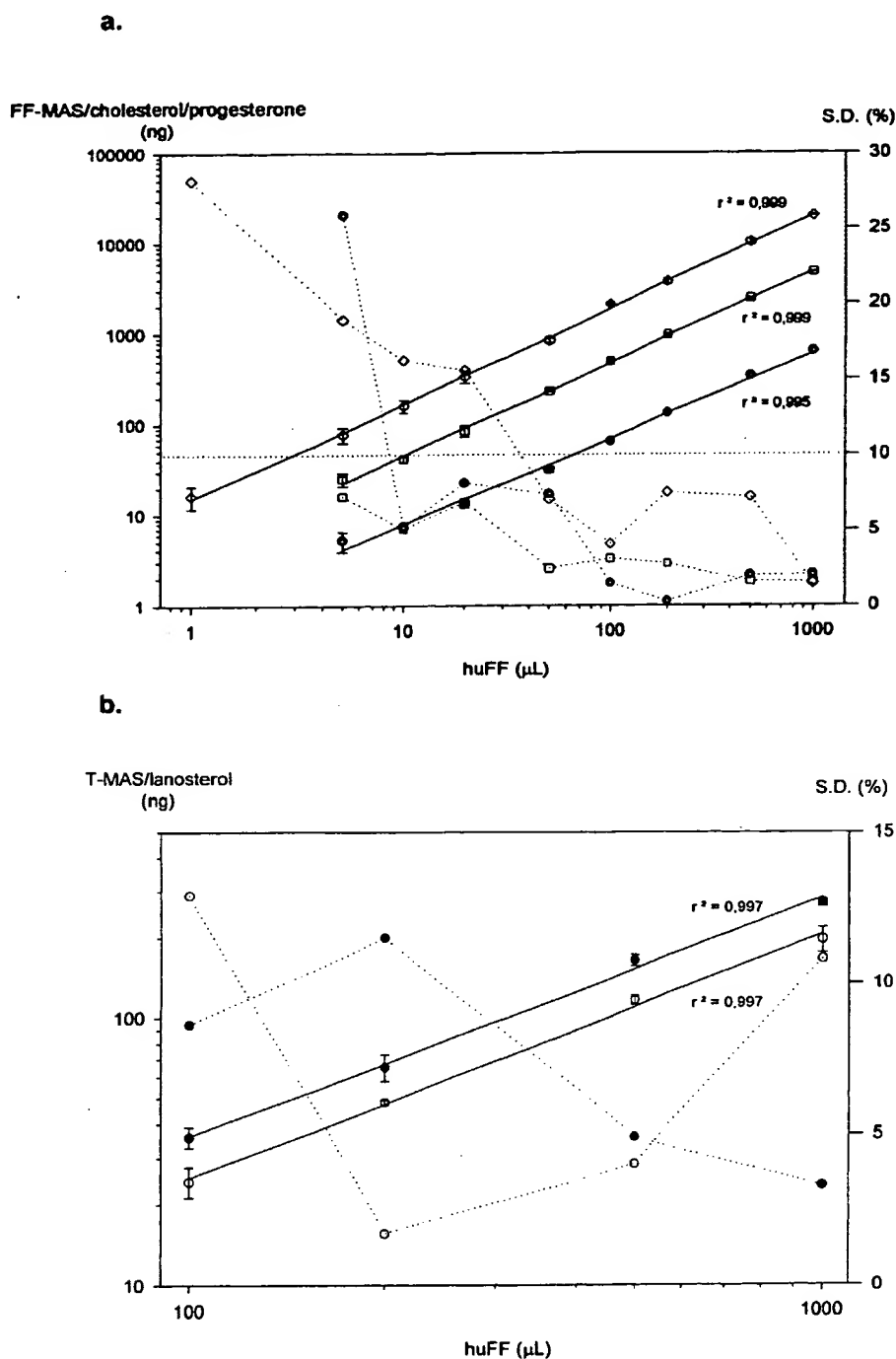


**Figure 3.** (a) Quantitation of FF-MAS (248.8 nm), free cholesterol (200 nm) and progesterone (234.7 nm) in huFF at the indicated wavelengths. A batch of huFF was diluted in PBS and assayed as described. A maximum standard deviation of 10% is indicated (dashed line). (b) Quantitation of lanosterol (200 nm) and T-MAS (200 nm) in huFF based on an additional reversed-phase separation. Each point represents four replicates with the standard deviations shown as error bars. The samples were treated in four individual sets and the variation represents inter-assay variation.

**Table 1. Limit of detection (LOD) and limit of quantitation (LOQ) for all analytes. A minimum signal-to-noise ratio of 3 was assumed in order to produce a significant assay signal.**

|  | LOD (ng/mL) | LOQ (ng/mL) |
|---|---|---|
| FF-MAS[a] | 1.5 | 4 |
| T-MAS[b] | 7 | 23 |
| Lanosterol[b] | 7 | 23 |
| Cholesterol[a] | 35 | 120 |
| Progesterone[a] | 1 | 3 |

[a] Estimated values (no empty matrix available).
[b] Signal-to-noise ratio greater than 3 for LOD and greater than 10 for LOQ.

**Table 2. Re-analysis of samples of huFF performed in order to evaluate the efficiency of the extraction procedure. Values are given as percentage analyte compared to the amount found in the first round of analysis. Values should be compared to values for spike recovery (Fig. 4).**

|  | 1000 μL sample | 500 μL sample | 200 μL sample |
|---|---|---|---|
| FF-MAS | 0.20%[a] | n.d. | n.d. |
| T-MAS | n.d. | n.d. | n.d. |
| Lanosterol | n.d. | n.d. | n.d. |
| Cholesterol | 0.92 ± 0.31% | 0.91%[b] | n.d. |
| Progesterone | 7.4 ± 0.78% | 6.3 ± 2.6% | 4.4 ± 1.70% |

[a] Only one sample had a detectable level of FF-MAS.
[b] Only three samples had a detectable level of cholesterol.
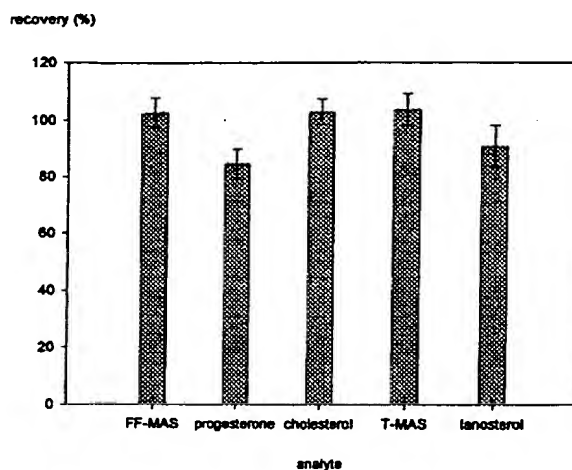


**Figure 4.** Spike-recovery analysis; 1000 μL huFF were added to approximately 100% of the analytes in n-heptane before sample treatment. Recovery rates were calculated on the basis of standards and spike mixtures added to pure PBS. Each point represents eight replicates with the standard deviations shown as error bars.

evaluation of MAS accumulation up to the approach of ovulation, due to the concurrent quantitation of progesterone.
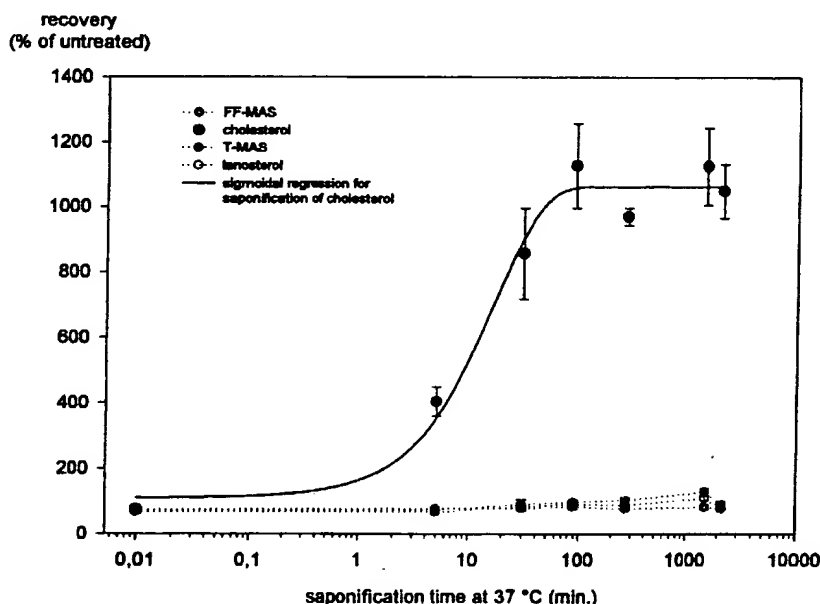


**Figure 5.** Saponification of huFF. Cholesterol and 4,4 dimethylsterols were measured in 200 μL huFF treated in 0.3 M KOH in EtOH at 37°C for the indicated period of time (abscissa) before analysis as above. Of the four analysed sterols only cholesterol caused increasing values with saponification time, indicating that the assayed 4,4-dimethyl sterols are not esterified in huFF in contrast to cholesterol. Values for the amount of free cholesterol in relation to time are fitted to a sigmoid 4-parameter function (solid line).

## Acknowledgements

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